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14. ABSTRACT Kaiso is a member of the POZ-ZF family of transcription factors, first identified as a binding partner for the p120ctn cell adhesion co-factor. Previous work in our laboratory showed that Kaiso was misexpressed in ~40% of human breast tumors and we also identified cyclin D1 as a putative Kaiso target gene. This project elucidated the mechanism by which Kaiso regulates cyclin D1 expression and the effects of this on breast cell tumorigenesis. Using minimal promoter assays, electrophoretic mobility shift assays and immunoblotting we showed how Kaiso regulates cyclin D1 expression. Our work to date demonstrates that Kaiso regulates cyclin D1 transcription through its binding properties (by binding to both sequence specific TCCTGCNA and methylated CpG sites within the promoter). We also demonstrated that Kaiso and p120ctn can modulate canonical Wnt signaling and activation of cyclin D1.					
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The Role of the POZ-ZF Transcription Factor Kaiso in Breast Cell Proliferation and Tumorigenesis

INTRODUCTION

The majority of fatal and invasive human tumors derive from epithelial origin. These tumors are characterized by their reduced intercellular adhesion, and increased mobility to secondary sites [1]. One complex that is essential for epithelial cell adhesion integrity and function is the E-cadherin-catenin complex. Malfunction of this complex has been implicated in ~50% of metastatic human carcinomas [1, 2]. E-cadherin is a transmembrane cell adhesion molecule that facilitates cell-cell adhesion via calcium-dependent homophilic interactions with other E-cadherin molecules on adjacent cells [3]. In the cytosol E-cadherin is anchored to the actin cytoskeleton via interactions with the catenin proteins (α -, β -, γ -, and p120^{ctn}). β -catenin binds to the catenin binding domain (CBD) of E-cadherin and links E-cadherin to the actin cytoskeleton via α -catenin [4]. E-cadherin also interacts with p120^{ctn} (hereafter p120) at its juxtamembrane domain (JMD) and this increases cell-cell adhesion strength [5]. Although there is a correlation between tumor invasion, metastatic characteristics and the integrity of the E-cadherin/catenin complex, some tumors retain normal expression of E-cadherin and the classical catenins (β - and γ -). This implicates other catenins, for example p120, as important factors in tumor progression. In an effort to further decipher p120 function a yeast-two hybrid screen using p120 as bait was performed. This identified the POZ-ZF protein Kaiso as a p120-specific binding partner [6]. Kaiso is a dual specificity transcription factor that can bind to both a sequence specific Kaiso binding site (KBS) TCCTGCNA and methylated CpG residues [7, 8]. Studies from our lab here found that Kaiso is misexpressed in ~40% of human breast tumors. This is consistent with the fact that POZ-ZF transcription factors have roles in cancer as either oncoproteins or tumor suppressors [6, 9, 10].

Several lines of evidence from our lab and others implicate Kaiso and p120 as modulators of Wnt/ β -catenin signaling whose malfunction is implicated in several human cancers. We found that a subset of Wnt/ β -catenin target genes, including *cyclin D1*, possessed two or more copies of the sequence specific KBS. This finding raises the possibility that Kaiso was a potential regulator of *cyclin D1*, a key cell cycle protein that is aberrantly expressed in human breast cancers [11-13]. The research performed by myself and the previous P.I. of this award, Ms. Abena Otchere revealed that Kaiso binds the *cyclin D1* promoter region *in vitro* and *in vivo* and represses the *cyclin D1* promoter. We also found whereas p120 activates a minimal *cyclin D1*-promoter that Kaiso inhibits β -catenin-mediated activation of the minimal *cyclin D1*-promoter. Furthermore, we found that overexpression or depletion of Kaiso in the MCF7 human breast cancer cell line resulted in a positive correlation of Cyclin D1 levels. These initial findings suggested that Kaiso and p120 may be involved in a signaling pathway, where Kaiso regulates genes involved in tumorigenesis and its binding partner p120 serves to modulate its function. These findings support our hypothesis that **Kaiso plays a role in breast cell proliferation and tumorigenesis via its regulation of *cyclin D1* expression.**

Ms. Abena Otchere, the original P.I of this grant, graduated and entered medical school approximately one year after the award was granted to her. The grant was subsequently awarded to me in September 2006 when I began my graduate studies in the laboratory of Dr. Juliet Daniel. Below is a summary of all the work performed and completed during the tenure of this grant (March 2005 to March 2008).

APPROVED STATEMENT OF WORK

Specific Aim 1: Examine Kaiso expression and function in human breast tumors.

GOALS	PROGRESS
Establish a correlation between Kaiso and Cyclin D1 expression in breast tumors.	Ongoing
Generate a Kaiso overexpression model system.	Complete
Determine the effects of Kaiso misexpression on breast cell growth and transformation.	Ongoing

Specific Aim 2: Characterize *cyclin D1* as a putative Kaiso target gene.

GOALS	PROGRESS
Determine if Kaiso interacts with the <i>cyclin D1</i> promoter in a methylation dependent manner.	Complete
Examine the effects of Kaiso misexpression on <i>cyclin D1</i> expression.	Complete

Specific Aim 3: Analyze catenin function in transcriptional regulation of *cyclin D1*.

GOALS	PROGRESS
Assess role of p120 ^{ctn} in modulating Kaiso-mediated transcriptional regulation of <i>cyclin D1</i> .	Ongoing
Determine if p120 ^{ctn} /Kaiso antagonize or synergize with β -catenin/TCF regulation of <i>cyclin D1</i> .	Complete

BODY

Specific Aim 1: Examine Kaiso expression and function in human breast tumors.

Aim 1.1: Establish a correlation between Kaiso and Cyclin D1 expression in breast tumors. The initial optimization of Kaiso and Cyclin D1 antibody concentrations was initially performed using murine breast tissue (**Figure 1, Appendix**). After obtaining human breast tissue for antibody optimization we found that Kaiso staining was not seen. Further optimization using higher antibody concentrations is currently underway. Analysis of 650 annotated breast tumor tissue samples will then be performed in collaboration with Dr. David Rimm at the Yale University Cancer Center using automated quantitative analysis (AQUA), an objective method of scoring developed by Dr. Rimm's laboratory.

Aim 1.2: Generate a Kaiso-overexpression model system. In order to create a human breast cancer Kaiso-overexpression cell line, MCF7 breast tumor cells were transfected with the pcDNA3 human Kaiso expression construct. The pcDNA3 plasmid contains a neomycin resistance gene which is used as a selectable marker for stable cell line creation (Invitrogen). I have successfully created this Kaiso overexpression cell line after screening many putative clones (**Figure 2, Appendix**). These cell lines along with Kaiso depleted cell lines, were used in experiments to determine the effect of Kaiso misexpression on Cyclin D1 protein levels (**Specific Aim 2.2, below**). Kaiso-overexpressing cell lines will also be used to complete **Specific Aim 1.3** which is to determine the effects of Kaiso misexpression on breast cell growth and transformation.

Aim 1.3: Determine the effects of Kaiso misexpression on breast cell growth and transformation. Due to time constraints this sub aim was unable to be completed in this time frame.

Outcome:

- 1. Optimization of Kaiso-specific monoclonal antibodies for immunohistochemistry.**
- 2. Generation of a Kaiso overexpression breast tumor stable cell line.**

Specific Aim 2: Characterize cyclin D1 as a putative Kaiso target gene.

Aim 2.1: Determine if Kaiso interacts with the *cyclinD1* promoter in a methylation-dependent manner.

A total of three potential Kaiso binding sites were identified within the *cyclin D1* promoter. They are located at -1118, +24 and +1050 with respect to the transcriptional start site (**Figure 3**). 30 bp oligonucleotides, which span each of these KBS sites, were created and Kaiso binding to these determined using electrophoretic mobility shift assays (EMSA). We found that purified Kaiso fusion constructs bound to the -1118 KBS oligonucleotide (**Figure 4**). Furthermore, when point mutations were introduced in this KBS site, binding was abolished (**Figure 4**). Unlike the -1118 KBS derived oligonucleotide, the +24 and +1050 KBS derived oligonucleotides did not bind to Kaiso fusion constructs in these experiments (**Figure 5**). As Kaiso has been shown to have dual DNA binding specificity the possibility existed that Kaiso could bind to these regions in a methylation-dependent manner [7]. To test this possibility the +24 and +1050 KBS derived oligonucleotides were methylated *in vitro* and tested for Kaiso binding in EMSAs. Kaiso fusion proteins bound to the methylated +24 KBS derived oligonucleotides but not to the +1050 KBS derived oligonucleotides (**Figure 6**). These data suggest that Kaiso may regulate the *cyclin D1* promoter through two mechanisms, via a KBS-specific site at the -1118 KBS site and a methylation-specific manner in the region containing the +24 KBS sequence. Kaiso binding to these two regions of the *cyclin D1* promoter was detected *in vivo* through chromatin immunoprecipitation assays (ChIP) (**Figure 7**).

Since Kaiso binds methylated CpG dinucleotides this led us to search the *cyclin D1* promoter region for CpG islands and we identified 10 potential CpG-dinucleotide pairs (i.e. CpG CpG) that may be recognized and bound by Kaiso (**Figure 3**). Eight oligonucleotides were created to span each of these sequences. These oligonucleotides were tested for their ability to bind Kaiso in both a methylation-dependent and independent

manner in EMSA experiments. Kaiso bound all eight of the methylated but not unmethylated oligonucleotides (**Figure 8**). It was shown that although Kaiso bound all of the methylated CpG CpG sequences, it did so with different affinities. To determine the relative affinity of Kaiso binding to the eight CpG-site oligonucleotides, an EMSA using all eight probes and GST-hKaiso ZF construct was performed. Kaiso bound CpG5 and CpG8 oligonucleotides with higher affinity than any other CpG site, suggesting that these sites were more physiologically relevant (**Figure 9**). These two strong binding sites (CpG5 & CpG8) were further tested for binding to the full-length wild-type Kaiso and Kaiso-zinc finger point mutants. The two strongest binding CpG sites bound to the full-length Kaiso-GST fusion protein (**Figure 10**) while the two weakest sites (CpG3 and CpG6) did not bind (data not shown). Cold-competition assays of the high affinity CpG5 and CpG8 sites revealed that excess cold probe outcompeted binding to these regions (**Figure 11**). This finding underscores the specificity of Kaiso-CpG binding.

To determine if Kaiso regulates *cyclin D1* expression, promoter reporter assays were performed in HeLa cells using a minimal *cyclin D1* promoter-luciferase reporter construct. These experiments clearly demonstrated that human Kaiso is a dose-dependent transcriptional repressor of the *cyclin D1* promoter (**Figure 12**). This was the first experimental evidence that Kaiso may indeed regulate *cyclin D1* transcript expression. Site-directed mutagenesis was used to create point-mutations in the two sequence-specific KBSs (-1118 and +24) located within the *cyclin D1* promoter fragment of the 1748CD1 construct. Unexpectedly, Kaiso was still able to repress luciferase expression under the control of the mutant KBS *cyclin D1* promoter constructs (**Figure 13**). Since Kaiso is a bi-modal transcription repressor that also recognizes and binds methyl-CpG, we postulated that Kaiso's effects on the *cyclin D1* promoter may be due to its association with methylated CpG sites in the *cyclin D1* promoter [7], in addition to the sequence-specific KBS.

To test this hypothesis we tested the effects of Kaiso on the 962CD1 luciferase reporter construct, which lacks the -1118 KBS and contains all putative CpG sites were analyzed. Kaiso repressed luciferase expression 2-fold (**Figure 14**), suggesting that indeed Kaiso's binding to methyl-CpG DNA sites contributes to its transcriptional repression of *cyclin D1*. To further test the effect of Kaiso-mediated methylation-specific repression of the *cyclin D1* promoter, we performed a series of promoter-reporter luciferase assays were performed following *in vitro* methylation of the 1748CD1 *cyclin D1* partial promoter-reporter construct. We found that when methylated, the 1748CD1 construct was fully repressed both in the presence and absence of ectopic Kaiso (**Figure 15**). This raised the possibility that endogenous Kaiso was repressing the 1748CD1 promoter.

Aim 2.2: Examine the effects of Kaiso misexpression on *cyclin D1* expression.

To determine the effects of Kaiso depletion on Cyclin D1 protein levels, an siRNA-mediated Kaiso knockdown stable MCF7 cell line was used. Cells were either serum starved or serum stimulated 24 hours prior to harvesting, and Kaiso and Cyclin D1 protein levels were ascertained by Western blot. Depletion of Kaiso resulted in a decrease in Cyclin D1 protein levels (**Figure 16**). These data indicate that Kaiso may positively regulate Cyclin D1 in this cell line.

In order to further confirm the results found in the depletion cell lines, cell cycle synchronization and re-stimulation was performed on both the Kaiso overexpression and depletion cell lines. Cells were grown to approximately 50% confluency prior to serum starvation for 24 hours. After 24 hours of serum starvation for cell cycle synchronization a 12 hour time course of serum stimulation was performed. The cells were harvested and tested for Cyclin D1 and Kaiso protein levels. Consistent with unsynchronized Kaiso-depleted cell lines, knockdown of Kaiso resulted in a decrease in Cyclin D1 expression (**Figure 17**). Furthermore, this trend was seen in all cell cycle stages. Interestingly, Kaiso levels seemed to decrease as the cell cycle progressed, being highest during serum starvation and decreasing as the cells were serum stimulated. In the MCF7 overexpression cell lines it was also seen that Kaiso overexpression resulted in an increase in Cyclin D1 expression. In summary, both the Kaiso overexpression and depletion MCF7 cell lines showed that Kaiso and Cyclin D1 exhibit a positive correlation with respect to protein expression levels. These data were unexpected since the luciferase-reporter assays performed in HeLa cells suggested that Kaiso represses the *cyclin D1* promoter. The possibility exists that Kaiso's dual mechanism of DNA binding also represents different transcriptional regulatory functions. As methylated DNA binding is associated with DNA repression, this may be the

mechanism of regulation seen in HeLa cells. As the *cyclin D1* promoter in MCF7 cells is hypomethylated [14] this would suggest that Kaiso's regulation of *cyclin D1* in this cell line is independent of methylation and is sequence specific. This would be consistent with the fact that Kaiso is a transcription factor which along with transcriptional repression functions has also been shown to transcriptionally activate the neuromuscular gene *rapsyn* [15].

Outcome:

1. Kaiso binds the *cyclin D1* promoter *in vitro* and *in vivo*.
2. Kaiso regulates *cyclin D1* promoter via sequence specific and methyl CpG sites.
3. There is a positive correlation between Kaiso and Cyclin D1 levels in MCF7 cells.

Specific Aim 3: Analyze catenin function in transcriptional regulation of cyclin D1.

Aim 3.1: Assess role of p120^{ctn} in modulating Kaiso-mediated transcriptional regulation of cyclin D1.

To analyze the effects of p120 overexpression alone on the *cyclin D1* promoter, promoter-luciferase assays were performed. p120 activated *cyclin D1* expression (**Figure 18**), however repeated experiments could not definitely conclude that p120 relieves Kaiso-mediated repression of the promoter. Together these results suggest that p120 activation of the *cyclin D1* promoter may be independent of its interaction with Kaiso, and that the p120 transcription activation effects may be due to other intracellular signaling pathways that p120 is involved in, e.g. p120 regulation of the RhoA family of GTPases [16].

Aim 3.2: Determine if p120^{ctn}/Kaiso antagonize or synergize with β -catenin/TCF regulation of cyclin D1.

Since the p120/Kaiso interaction is reminiscent of the β -catenin/TCF interaction, and β -catenin/TCF are well-documented transcriptional regulators of *cyclin D1*, I investigated whether Kaiso and p120 synergize or antagonize with β -catenin/TCF regulation of *cyclin D1* using luciferase assays. Kaiso inhibited β -catenin-mediated activation of the *cyclin D1* promoter while p120 enhanced this activation (**Figure 19**). This exciting finding corroborates previous studies by us and our collaborator (Dr. Pierre McCrea) that Kaiso and p120 are indirect regulators of canonical Wnt signaling [17, 18].

To determine the effect of p120 on Kaiso's ability to inhibit β -catenin-mediated activation of the *cyclin D1* promoter, I performed promoter-luciferase assays using the 1748CD1 *cyclin D1* promoter- reporter plasmid, Kaiso, p120 and β -catenin overexpression vectors in HeLa cells. Due to inefficient transfection and overexpression (as determined by western blot) of the β -catenin overexpression plasmid the results obtained were inconclusive and need to be repeated. I am currently in the process of optimizing transfection efficiency of the β -catenin overexpression plasmid and will repeat the luciferase assay.

Outcome:

1. p120 activates the *cyclin D1* promoter.
2. Kaiso inhibits β -catenin-mediated activation of *cyclin D1*.
3. p120 enhances β -catenin-mediated activation of *cyclin D1*.

KEY RESEARCH ACCOMPLISHMENTS

1. Generation of a MCF7 breast tumor stable Kaiso-overexpression cell line.
2. Kaiso binds and regulates *cyclin D1* by dual-specificity mechanisms.
3. Kaiso and Cyclin D1 levels have a positive correlation in MCF7 cells.
4. Kaiso and p120 modulate canonical Wnt/ β -catenin target gene *cyclin D1*.

REPORTABLE OUTCOMES

Journal Articles:

1. **Manuscript in preparation.** Michelle I. Anstey, Abena A. Otchere, Kevin F. Kelly, and Juliet M. Daniel. *Kaiso is a transcriptional regulator of the cyclin D1 gene.*

Abstracts (presenters*):

1. **Abena A. Otchere*** and Juliet M. Daniel. *The cell cycle regulator cyclin D1 is a target gene of the POZ-ZF transcription factor Kaiso.* New England Biolabs Annual Symposium. (May 2005) Toronto, Ontario. **(Winner, Best Poster).**
2. Juliet M. Daniel, Kevin F. Kelly*, **Michelle I. Anstey***, Mai Almardini, Nickett S. Donaldson, and Monica Graham. *Crosstalk between p120^{ctn}/Kaiso and β -catenin/TCF signaling in human cancer.* Hamilton's Inaugural Health Research in the City. (January 2007). Hamilton, Ontario. **(Winner, Best Poster in the Cancer Division).**
3. **Michelle I. Anstey***, **Abena A. Otchere**, Kevin F. Kelly and Juliet M. Daniel. *Regulation of the Wnt-target genes cyclin D1 and matrilysin by Kaiso/p120^{ctn} signaling.* International Women's Day Research Conference. (March 2007). Hamilton, Ontario.
4. **Michelle I. Anstey***, **Abena A. Otchere**, Kevin F. Kelly and Juliet M. Daniel. *Kaiso-mediated transcriptional regulation of the cell cycle regulator, CyclinD1 and its effect on tumorigenesis.* Fifth Era of Hope Meeting. (June 2008). Baltimore, MD.

Conference Delegate

1. Edward A. Smuckler Memorial Workshop: Pathobiology of Cancer. Presented by the American Association for Cancer Research. Snowmass Village, Colorado. July 2005.

Degrees Obtained that are supported by this award:

- The former P.I. Ms. Abena Otchere received her M.Sc. degree in June 2006 after 1 year of support by this award.

Development of cell lines:

- Creation of a breast tumor, Kaiso overexpressing stable cell line.

LIST OF PERSONNEL WHO RECEIVED PAY FROM THIS AWARD

- Ms. Abena Otchere
- Ms. Michelle Anstey

CONCLUSIONS

Our tenure of this award has been very rewarding and productive. Our findings have indicated that Kaiso is a transcriptional regulator of the *cyclin D1* gene, potentially both activating and repressing *cyclin D1* expression depending on the cellular environment. We have also shown that the *cyclin D1* gene is potentially regulated by Kaiso through two different mechanisms; both sequence-specific and methylation-dependent manners. This would make *cyclin D1* a unique Kaiso target gene which differs mechanistically from Kaiso's other target genes such as *matrilysin* or *Wnt11* [18, 19]. Due to time constraints and technical difficulties we were unable to complete a few subaims namely analysis and comparison of Kaiso and Cyclin D1 protein levels in breast tumor tissue samples (Aim 1). I am optimistic that optimization of Kaiso antibodies for human tissue

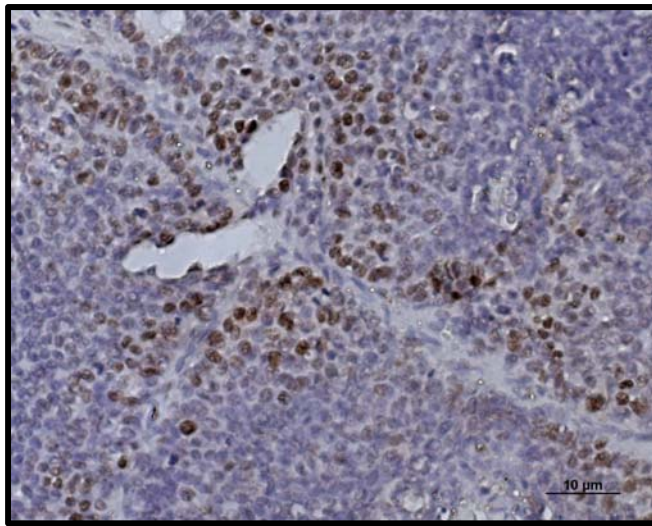
in immunohistochemistry will be accomplished in the next month. Assessment of Kaiso misexpression effects on cell proliferation and transformation (Aim 1) is another aspect that remains to be completed but we now have the reagents necessary to complete this aim in the near future. Our continued investigations will analyze the role that Kaiso plays directly in the cell biology of breast tumorigenesis. Our findings will contribute to understanding the role of Kaiso in breast tumorigenesis and may contribute to the future development of novel diagnostic, prognostic, and therapeutic tools in the fight against breast cancer.

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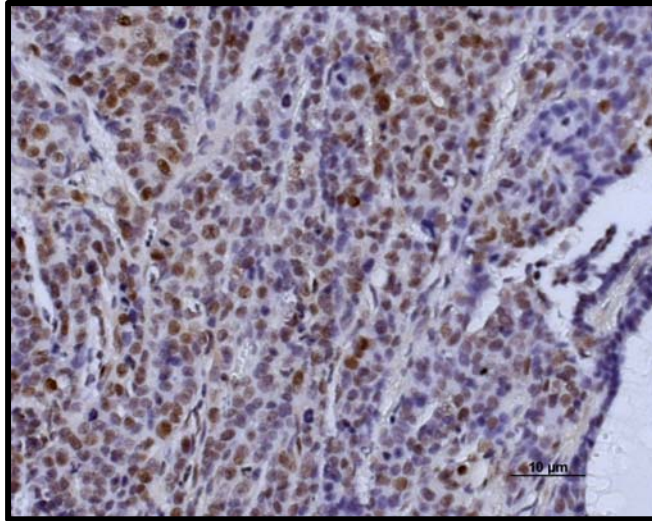
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APPENDIX

Anti-Kaiso



Anti-Cyclin D1



No primary
antibody

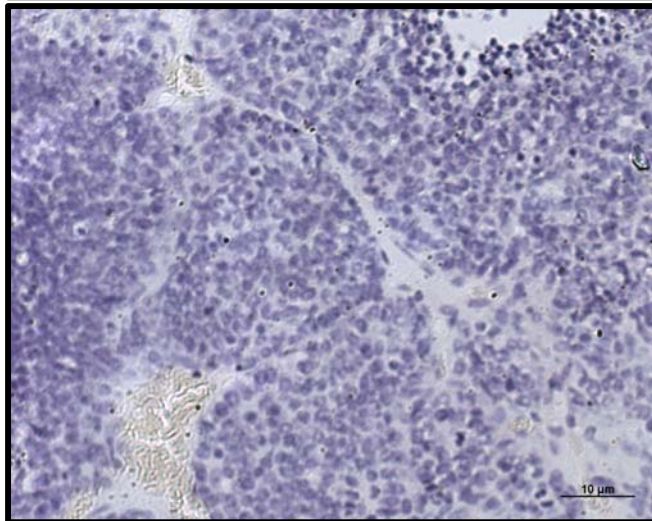


Figure 1: Immunohistochemistry of murine breast cancer tissue. To optimize both Cyclin D1 and Kaiso antibodies for use in immunohistochemistry, murine breast tumor samples were used. Kaiso polyclonal antibody was able to detect Kaiso at a dilution of 1:250. Cyclin D1 monoclonal antibody was able to detect Kaiso at a dilution of 1:10. The negative control slide was incubated with only a secondary antibody.

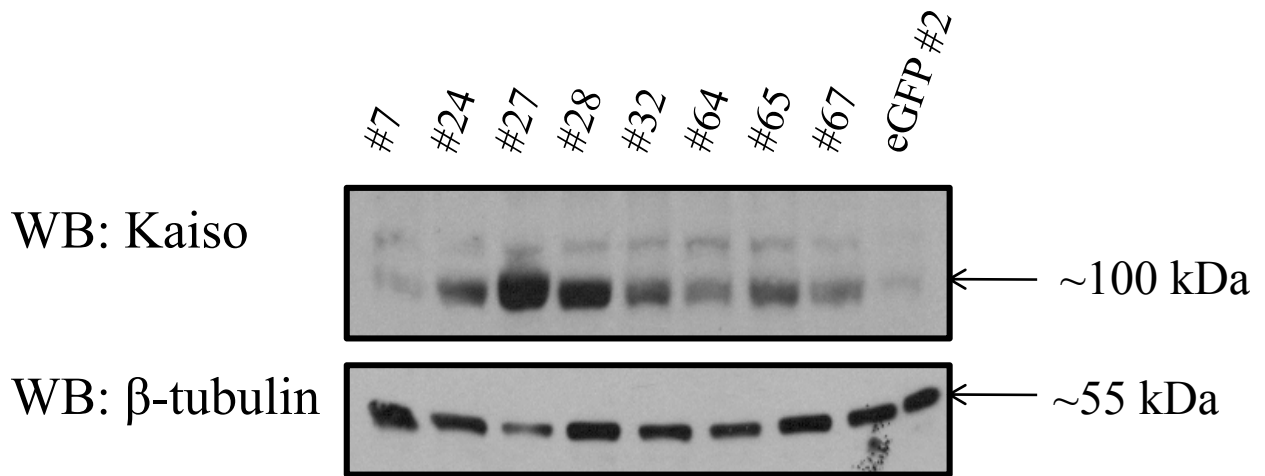


Figure 2: Generation of a Kaiso overexpression stable MCF7 cell line. The Kaiso overexpression plasmid, pcDNA3-hKaiso, which contains a neomycin resistance gene, was transfected into MCF7 cells. Cells were selected in G418 drug for 3 weeks and clones were isolated and tested for Kaiso overexpression. A number of positive clones were identified, with clone #27 and #28 having the highest expression of Kaiso. These cell lines were subsequently used in a number of experiments to assess the effects of Kaiso misexpression on Cyclin D1 expression. β -tubulin serves as a loading control.

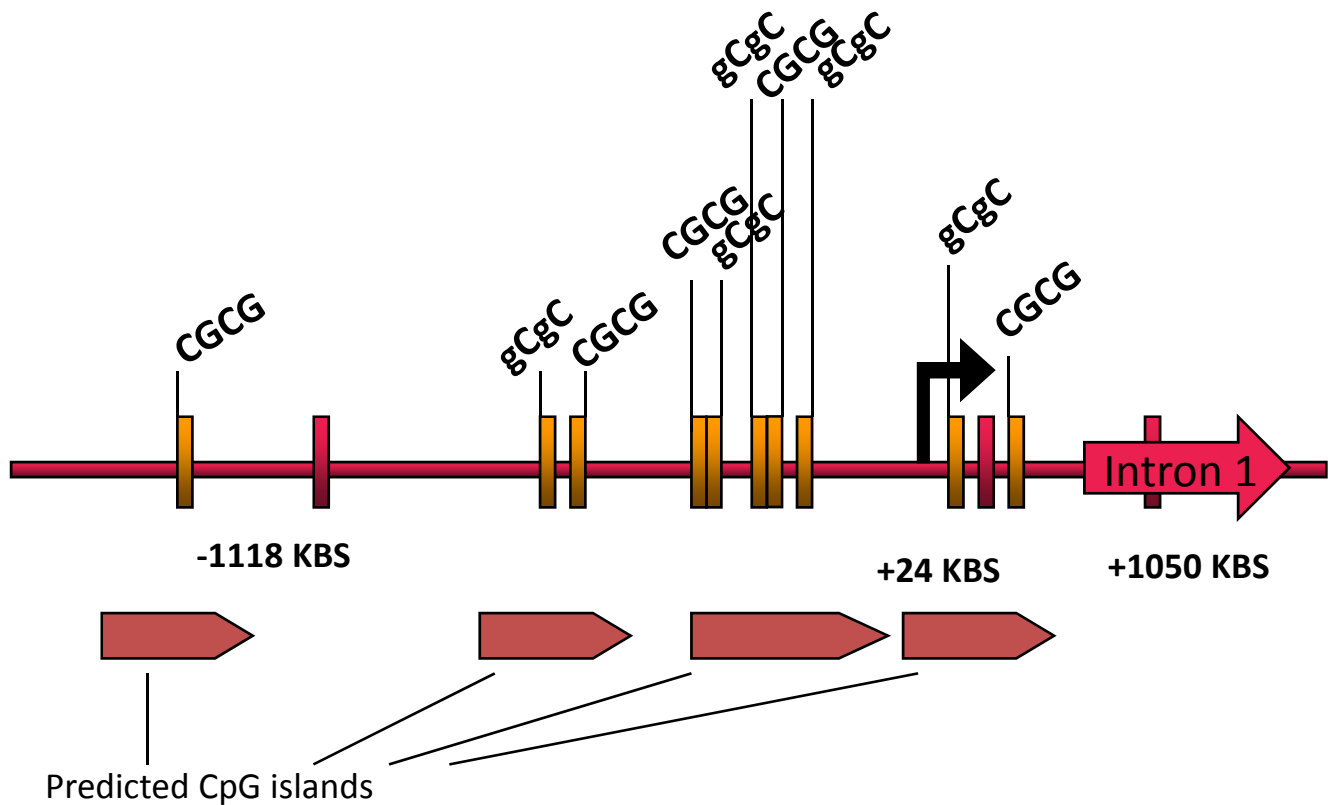


Figure 3: Schematic of *CCND1* promoter with KBS and CpG sites. The *CCND1* promoter contains three KBS sites located at -1118, +24, and +1050 relative to the transcriptional start site. Within the *CCND1* promoter there are also a number of CpG dinucleotide pairs (10 in total) that are located within predicted CpG islands.

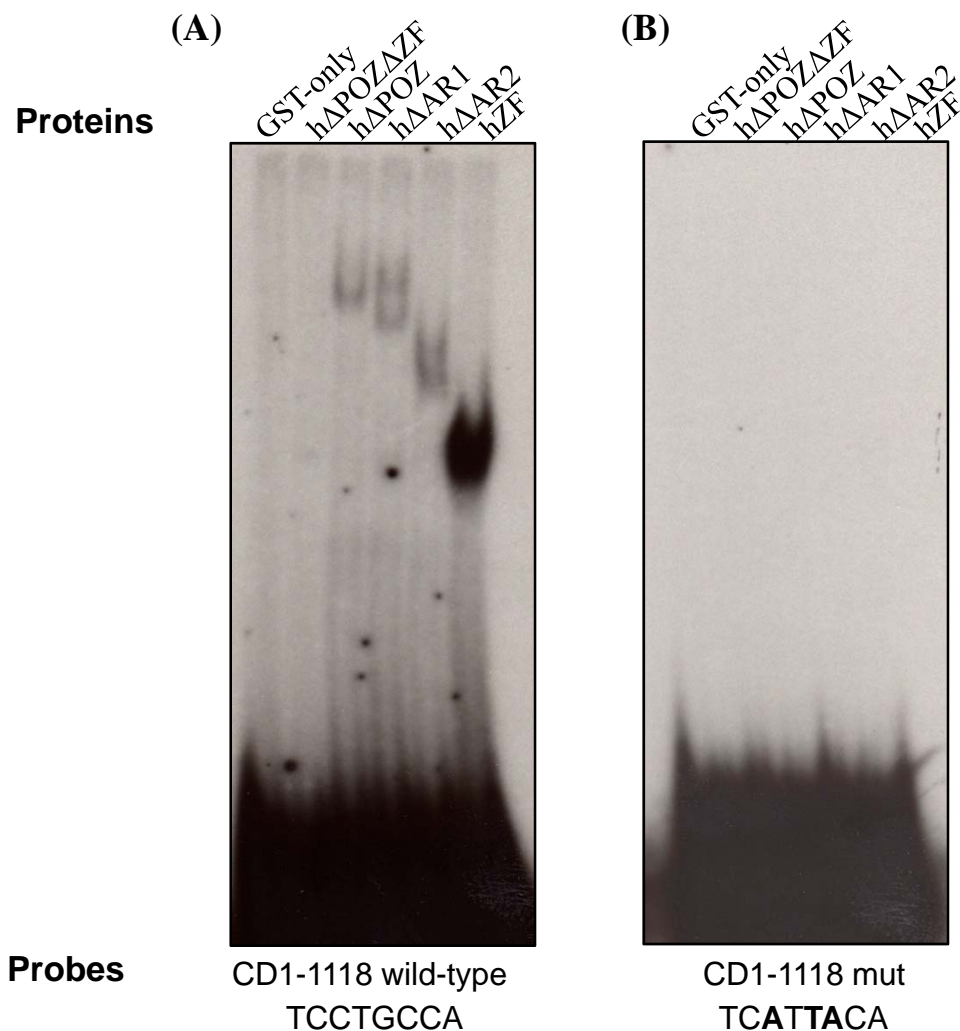


Figure 4: Kaiso binds to the -1118 KBS site of the *cyclin D1* promoter *in vitro*. Wild type and KBS mutated oligonucleotides spanning the -1118 KBS site were created. These oligonucleotides were then radiolabelled and incubated in a binding reaction with 250 ng of various GST-human Kaiso constructs. Negative controls include GST-tag alone, and the Kaiso protein lacking both the POZ and ZF domains. Other fusion constructs which are N-terminally deleted but still contain the ZF DNA binding domain and should bind DNA (hΔPOZ, hΔAR1, hΔAR2 and hZF). (A) The Kaiso proteins bound to the -1118 KBS oligonucleotide. (B) Binding to the oligonucleotide was abolished when point mutations were introduced in the KBS.

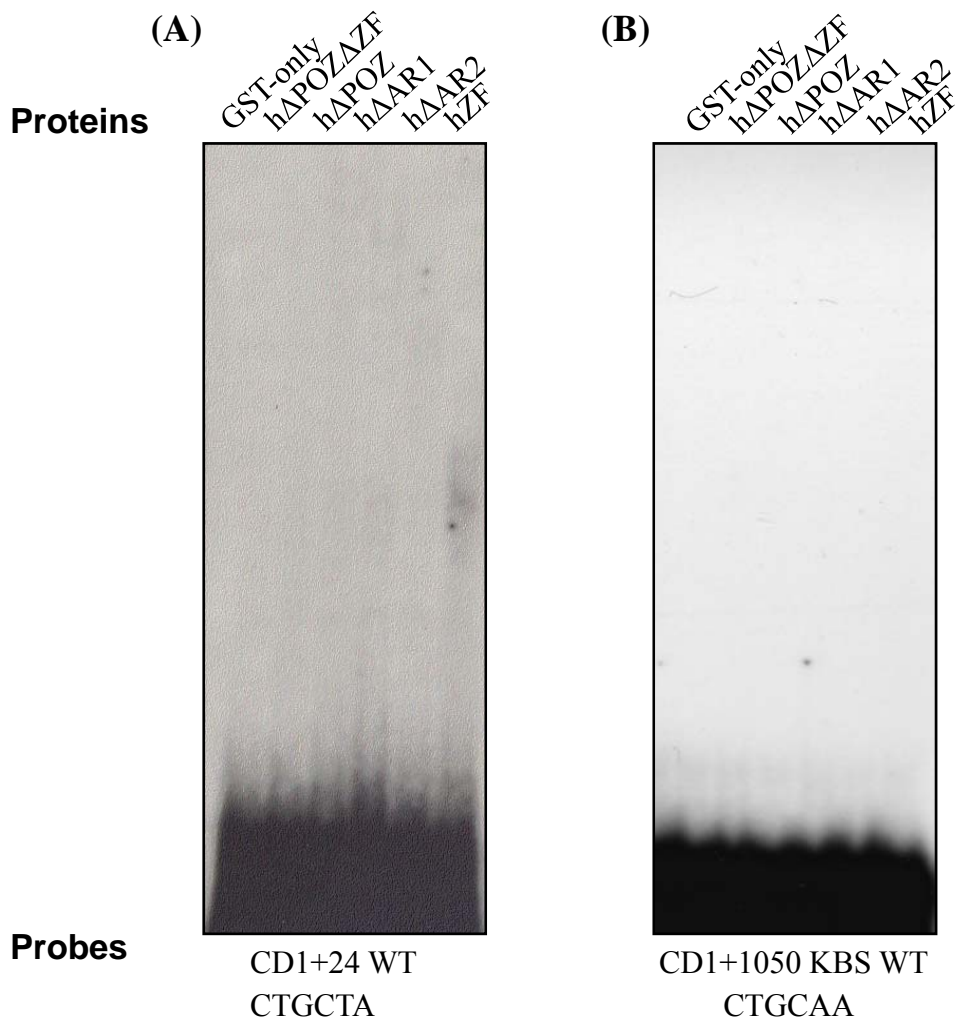


Figure 5: Kaiso does not bind to the +24 KBS or +1050 KBS site. Oligonucleotides spanning the +24 and +1050 KBS sites were created, radiolabelled and incubated in a binding reaction with 250 ng of various GST-hKaiso constructs. (A) The Kaiso-GST fusion proteins did not bind the +24 KBS region. (B) The Kaiso proteins did not bind +1050 KBS site.

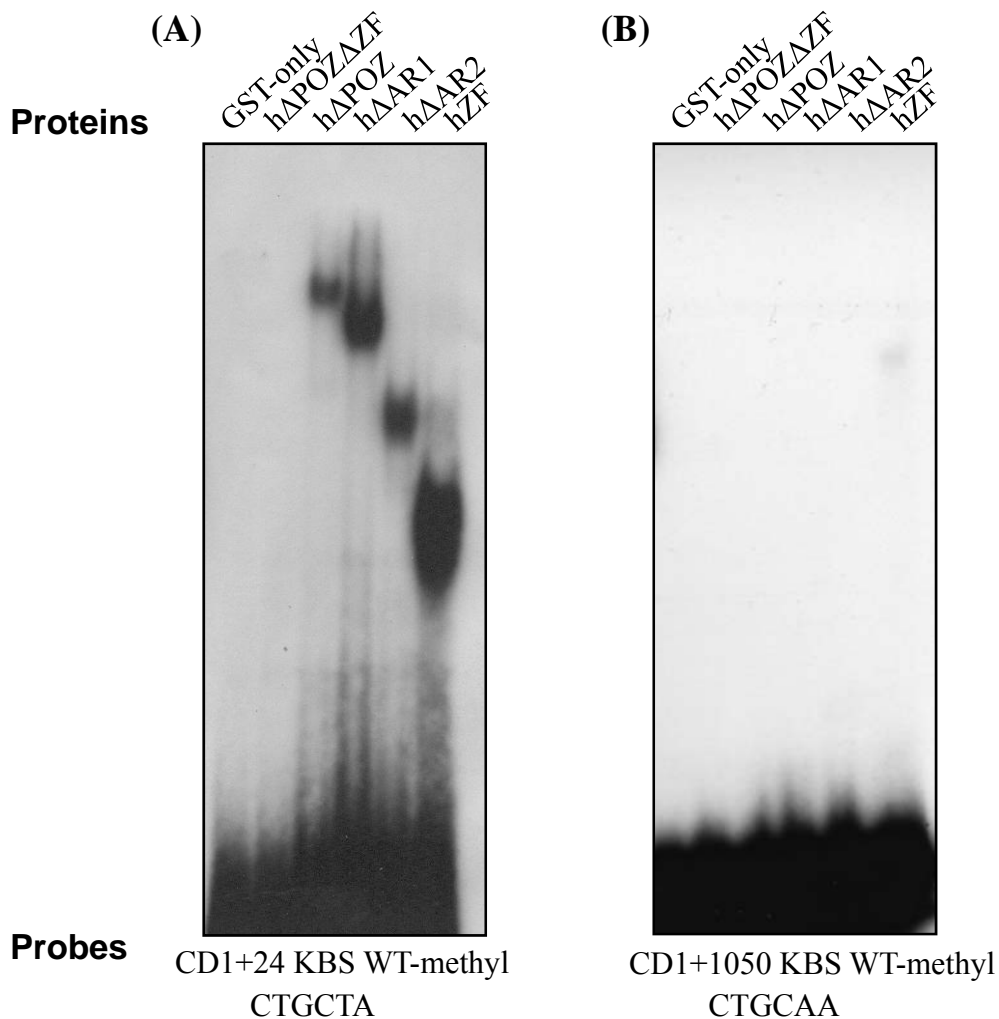


Figure 6: Kaiso binds the +24 KBS site when methylated but not the +1050 KBS. Oligonucleotides spanning the +24 and +1050 KBS site were methylated *in vitro*, radiolabelled and incubated in a binding reaction with 250 ng of various GST-hKaiso constructs. (A) The Kaiso-GST fusion proteins bound the +24 KBS region when methylated. (B) The Kaiso-GST fusion proteins did not bind to the methylated +1050 KBS site.

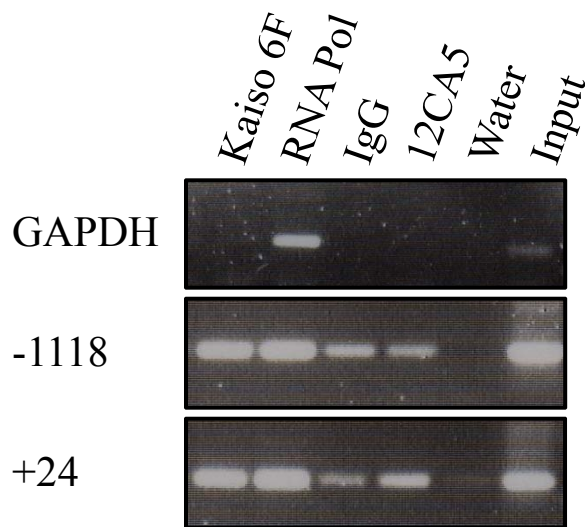


Figure 7: Chromatin Immunoprecipitation of Kaiso on *cyclin D1* promoter fragments. MCF7 breast tumor cells were used to isolate Kaiso-chromatin immunocomplexes. A Kaiso-specific mAb (6F) was used. Primers surrounding the -1118 KBS site on the *cyclin D1* promoter were used to successfully amplify chromatin associated with Kaiso. Primers targeting the +24 KBS site on *cyclin D1* promoter were also able to successfully amplify chromatin associated with Kaiso. Positive control antibody (RNA Pol II) derived immunocomplexes amplified the positive control primers from GAPDH, and to a lesser extent the negative control (IgG and 12CA5). Optimization is currently underway to reduce weak non-specific band seen in negative control lanes.

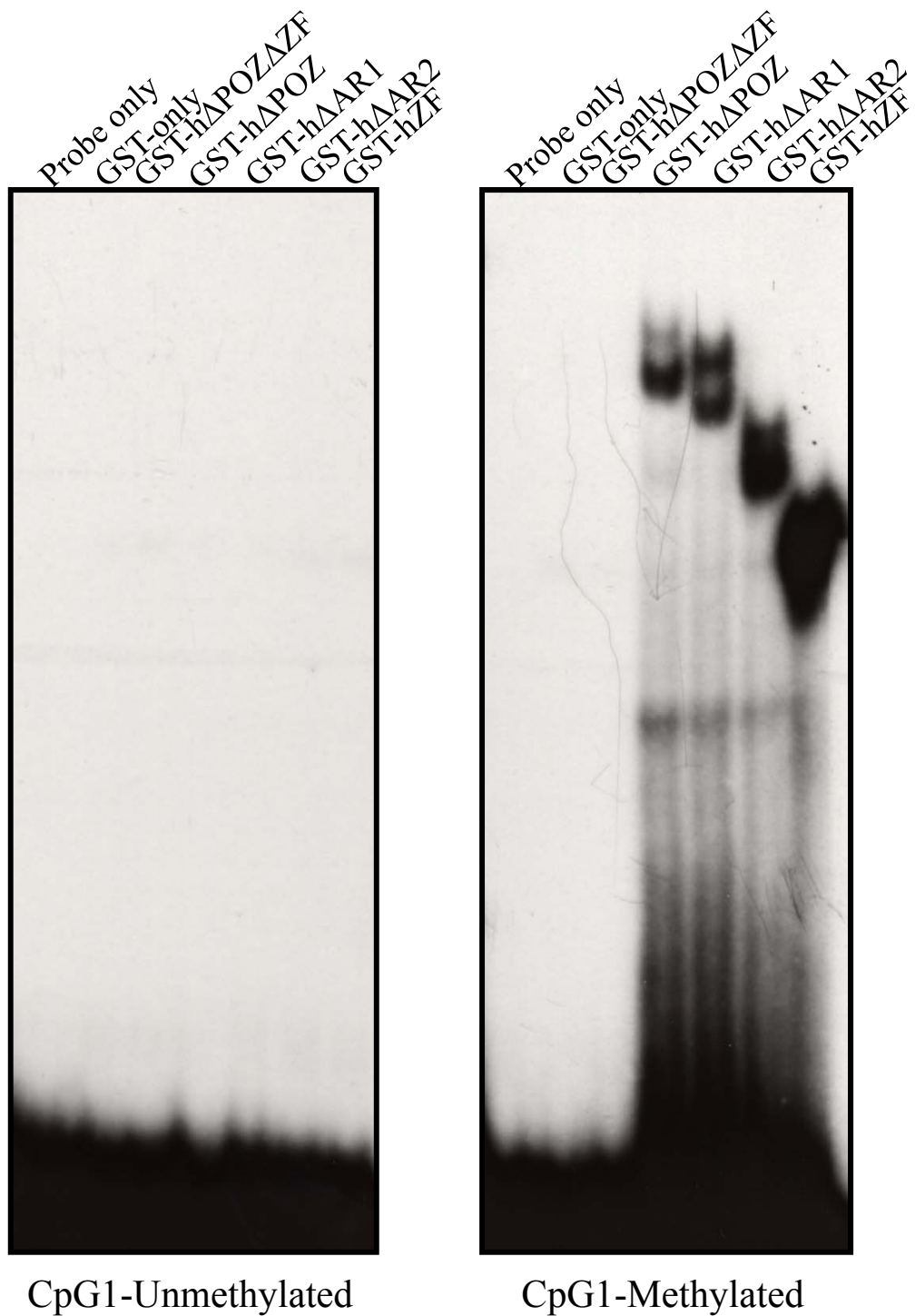


Figure 8: Kaizo binds to all methylated CpG oligomers in *cyclinD1* promoter. *CyclinD1*-promoter derived oligos (CpG1 to CpG8) were methylated *in vitro*, radiolabelled & incubated with GST-Kaizo proteins. Kaizo bound to methylated probes but not to unmethylated probes. The diagram below is an autoradiogram for oligonucleotide CpG1. Similar results were obtained for CpG2 to CpG8 (data not shown).

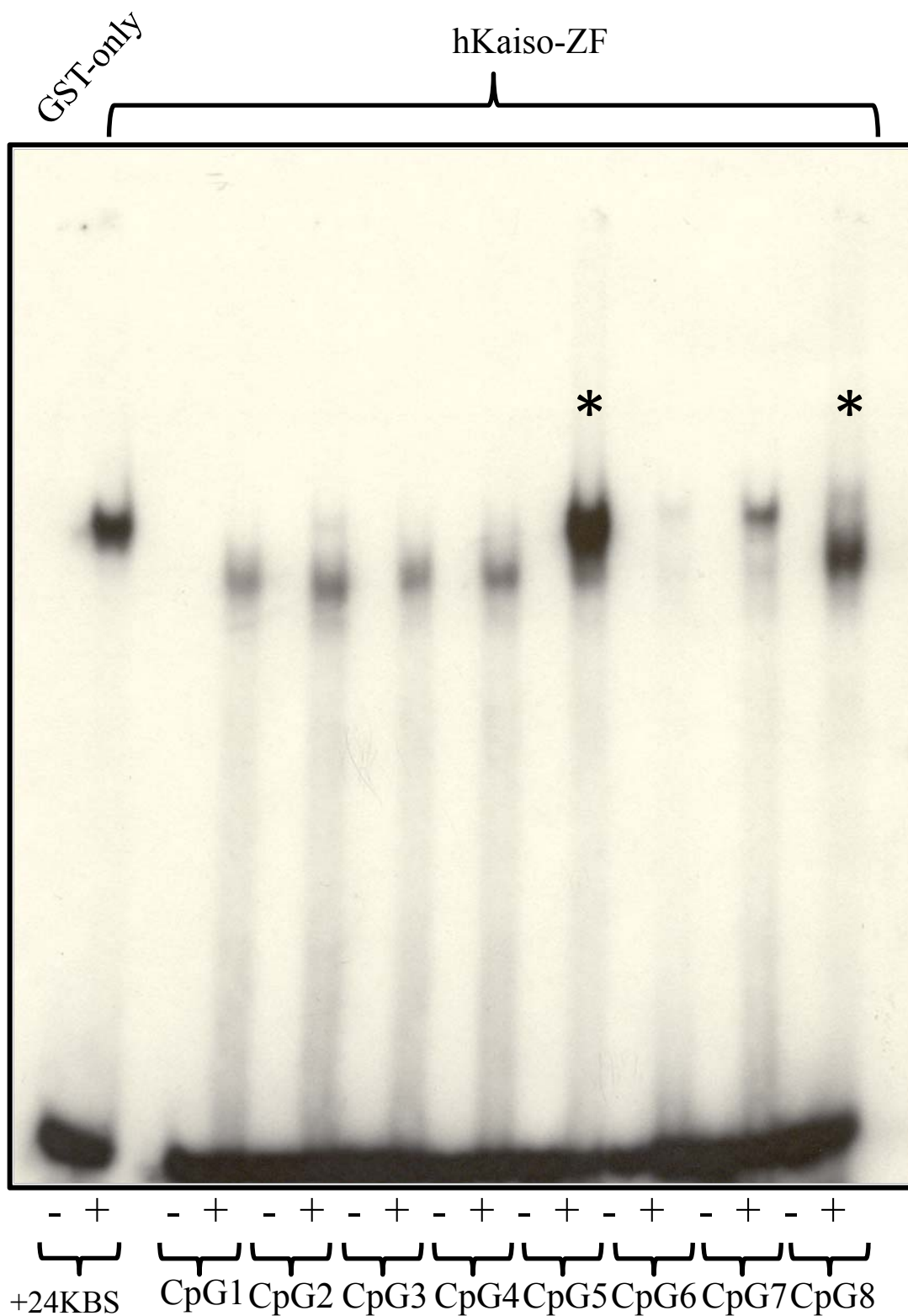


Figure 9: Kaiso binds the methylated CpG5 and CpG8 sites with the highest affinity. *CyclinD1*-promoter-derived oligos (CpG1 to CpG8) were methylated *in vitro*, radiolabelled & incubated with GST-hKaiso ZF protein. Kaiso bound to methylated probes but not to unmethylated probes. +; methylated, -; unmethylated.

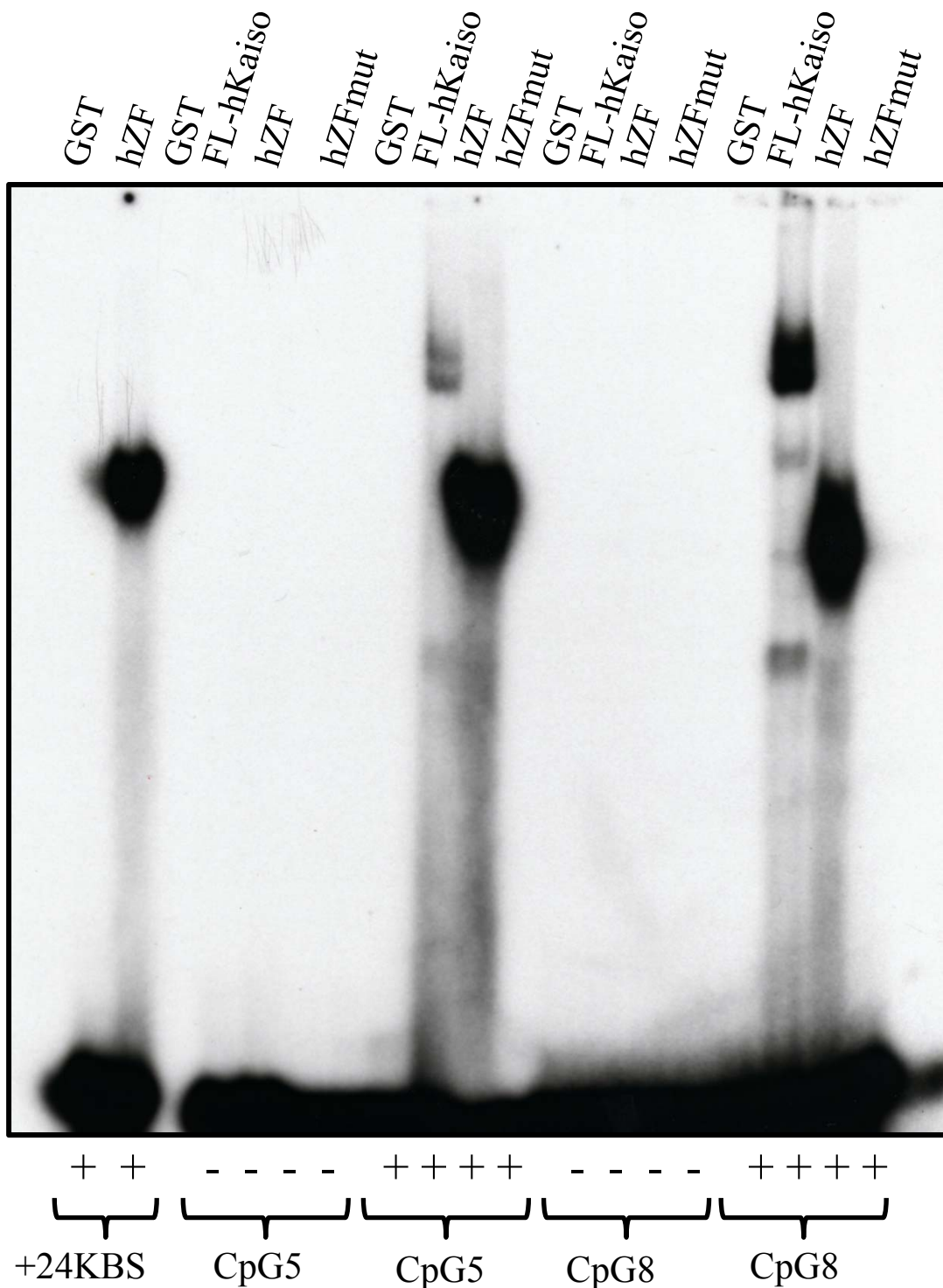


Figure 10: Full length Kaiso binds CpG5 and 8. Oligos CpG5 and CpG8 were methylated *in vitro*, radiolabelled & incubated with full-length GST-hKaiso, GST-hKaiso ZF and GST-hKaiso ZF mutant. Full-length Kaiso bound to CpG5 and CpG8 but the ZF mutant protein did not. Full length binds less than the ZF construct because the POZ domain is believed to sterically hinder DNA binding of POZ-ZF proteins *in vitro*. +; methylated, -; unmethylated.

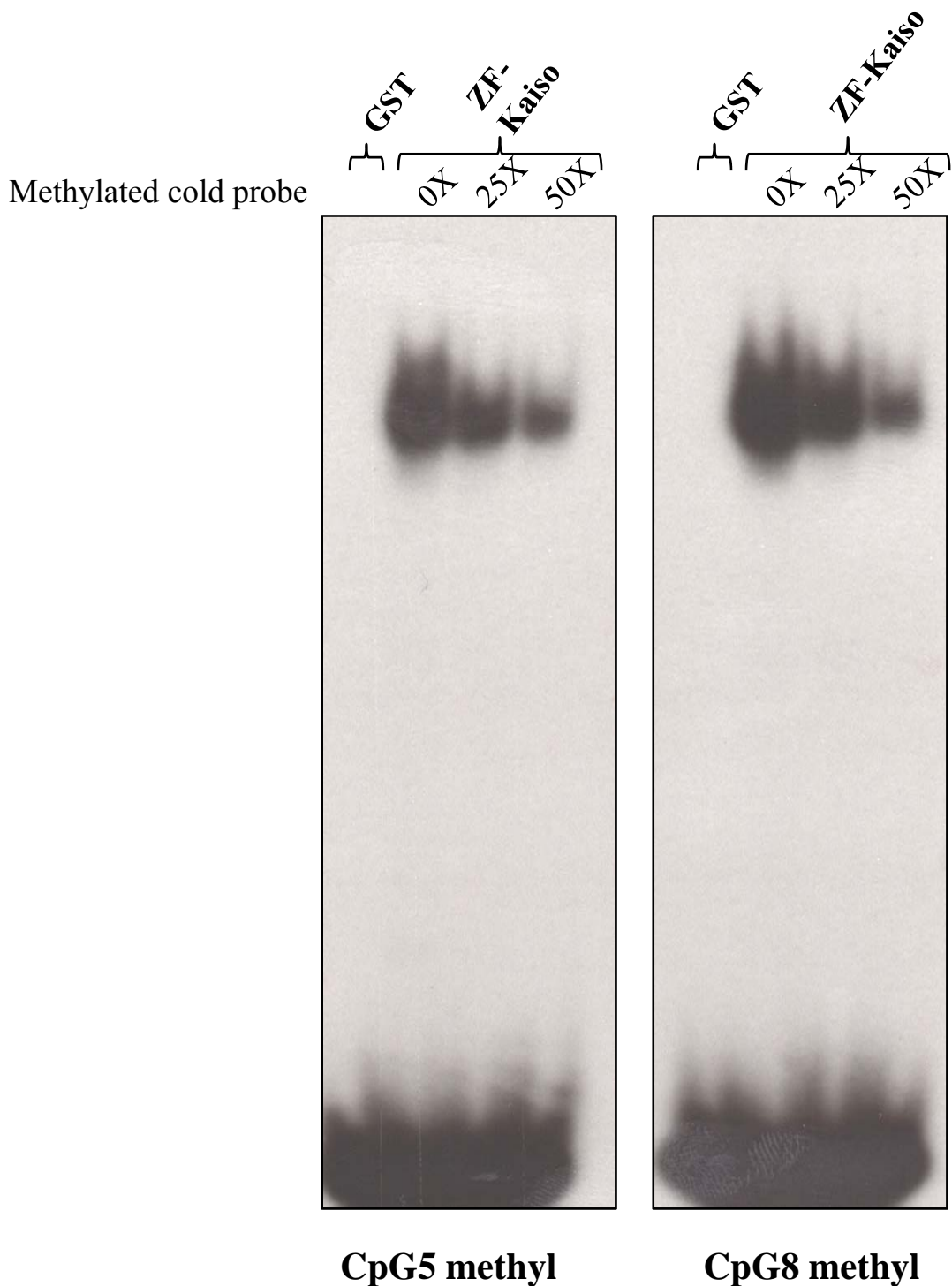


Figure 11: Kaiso specifically binds to the CpG5 and CpG8 regions when methylated. *cyclin D1*-promoter-derived CpG5 and CpG8 oligonucleotides were methylated *in vitro*, radiolabelled and incubated with GST-Kaiso proteins. Cold probe was added to the reactions at 25X and 50X excess labelled probe. Methylated cold probe was able to outcompete the binding of the GST-Kaiso ZF and full-length protein. As a negative control GST protein alone was tested.

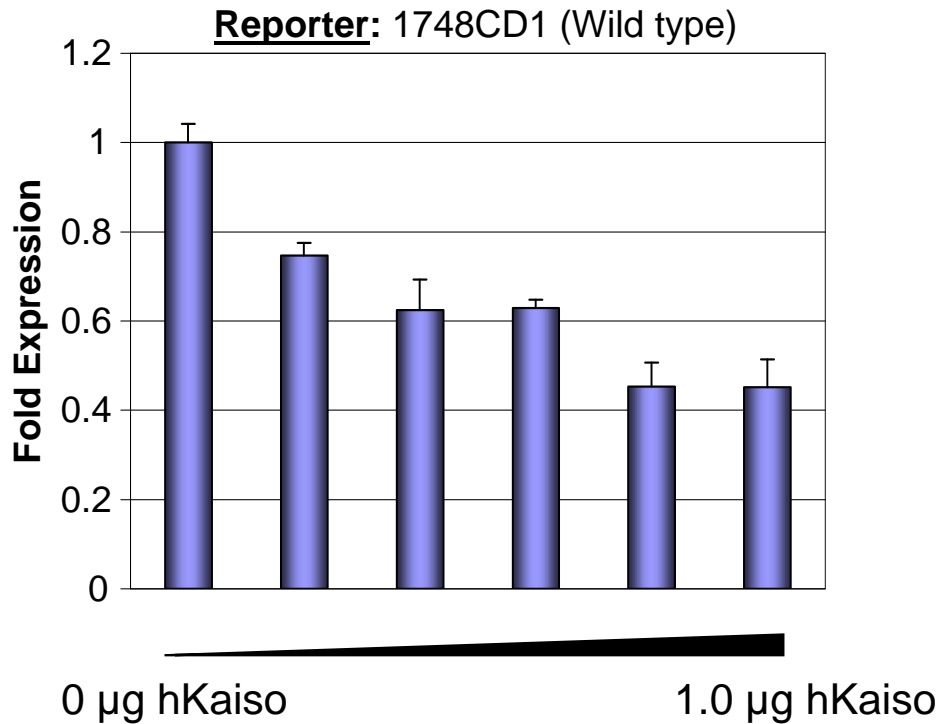


Figure 12: Kaiso represses transcription from the *cyclin D1* promoter in a dose-dependent manner. The 1748CD1 minimal *cyclin D1* promoter construct was co-transfected with increasing amounts of pcDNA3-Kaiso expression vector in HeLa cells. Kaiso overexpression in this system resulted in the dose-dependent repression of luciferase expression.

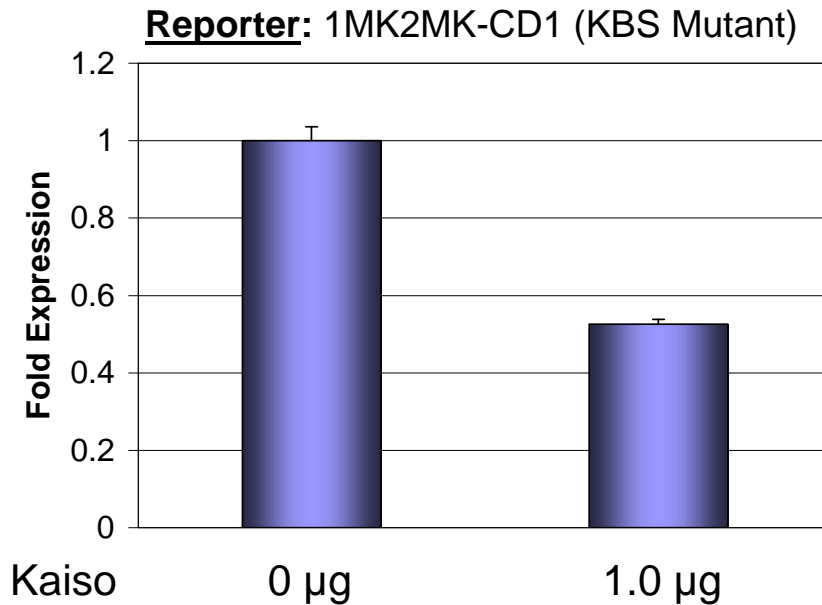


Figure 13: Kaiso represses luciferase expression from KBS point mutant *cyclin D1* promoter constructs. Kaiso was co-transfected along with the 1MK2MK-CD1 KBS mutant partial *cyclin D1* promoter construct which contains a single base pair point mutation in each of the two KBS located within the 1748CD1 construct. Unexpectedly Kaiso repressed luciferase expression 2-fold under the control of the KBS mutant *cyclin D1* promoter. This suggested an alternate mechanism of Kaiso mediated repression.

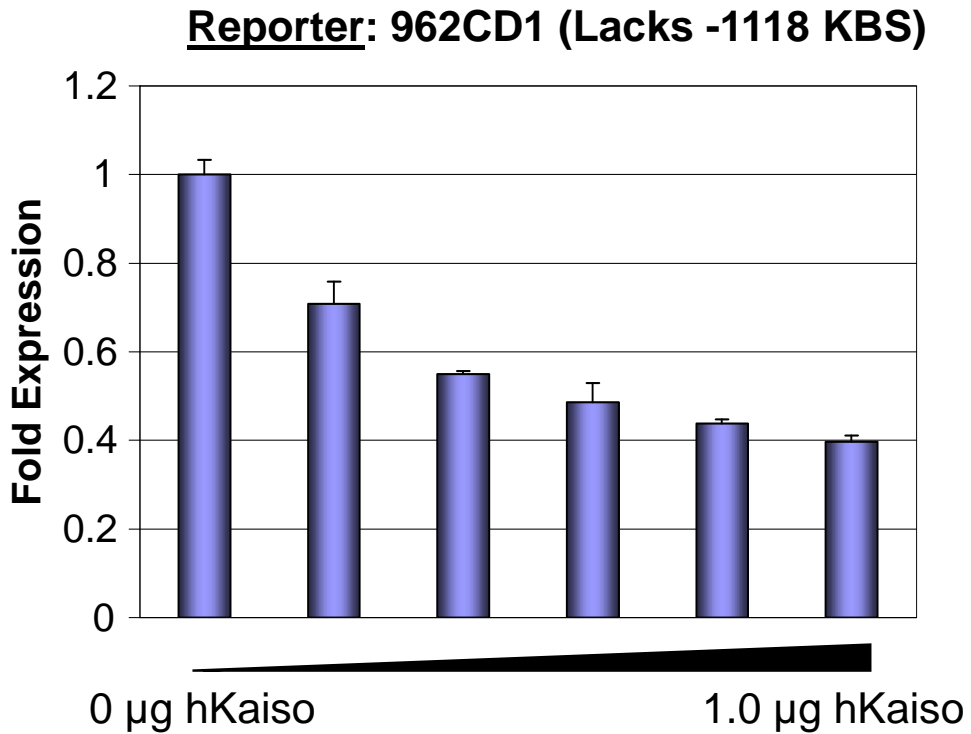


Figure 14: Kaiso represses luciferase expression from 962CD1 reporter which lacks -1118 KBS. Luciferase assays using the 962CD1 partial *cyclin D1* promoter construct, which lacks the -1118 KBS but contains putative CpG islands demonstrated a dose-dependent repression of luciferase expression by Kaiso to a maximum repression of 2-fold.

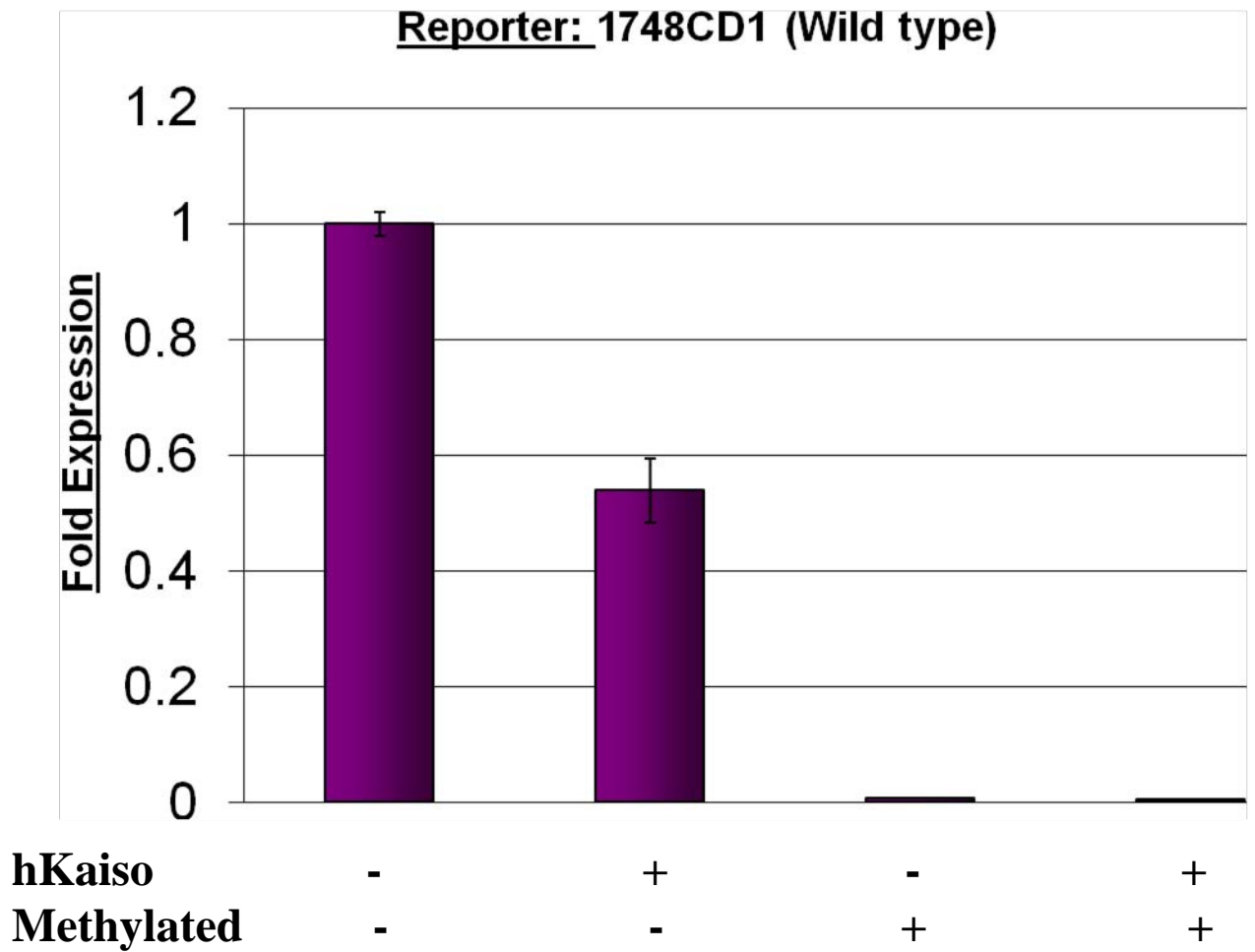


Figure 15: *In vitro* methylated 1748CD1 is completely repressed in the presence or absence of exogenous Kaiso. 1748CD1 partial *cyclin D1* promoter reporter was methylated *in vitro* using *Sss. I* methylase, and effects of Kaiso on the minimal promoter was assessed. The methylated *cyclin D1* promoter was completely repressed in presence or absence of ectopic Kaiso.

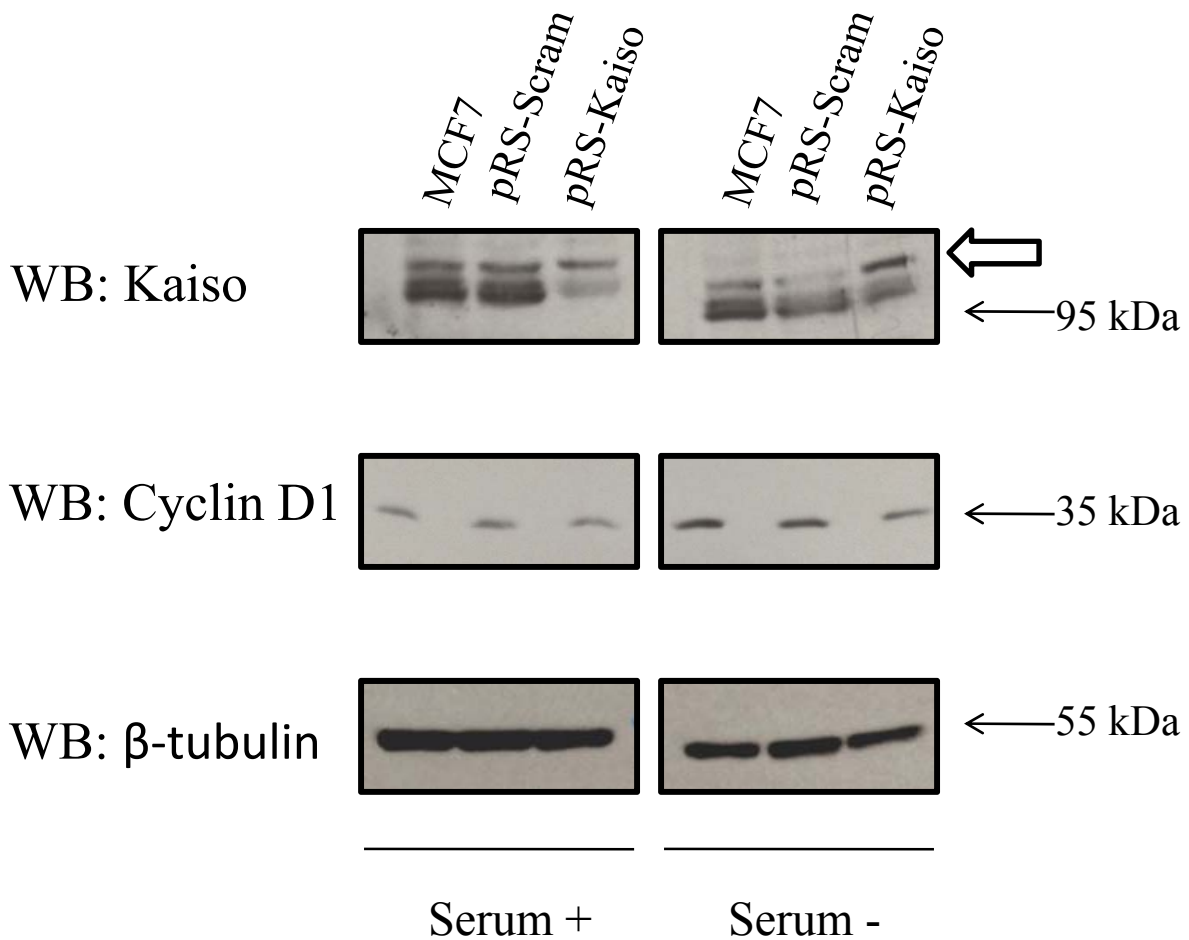


Figure 16: Knockdown of Kaiso in MCF7 cells causes a decrease in Cyclin D1 protein expression levels. MCF7 parental and siRNA Kaiso knockdown stable cell lines were subjected to serum starvation or serum stimulation. Kaiso and Cyclin D1 levels were assessed using WB and it was found that decreased Kaiso protein levels correlate with decreased Cyclin D1 levels. Kaiso and Cyclin D1 protein levels seem unchanged in the MCF7 siRNA Scrambled Kaiso control. Higher molecular mass band seen on the blots above the characteristic Kaiso doublet is a non-specific band which is not Kaiso (marked by open arrow).

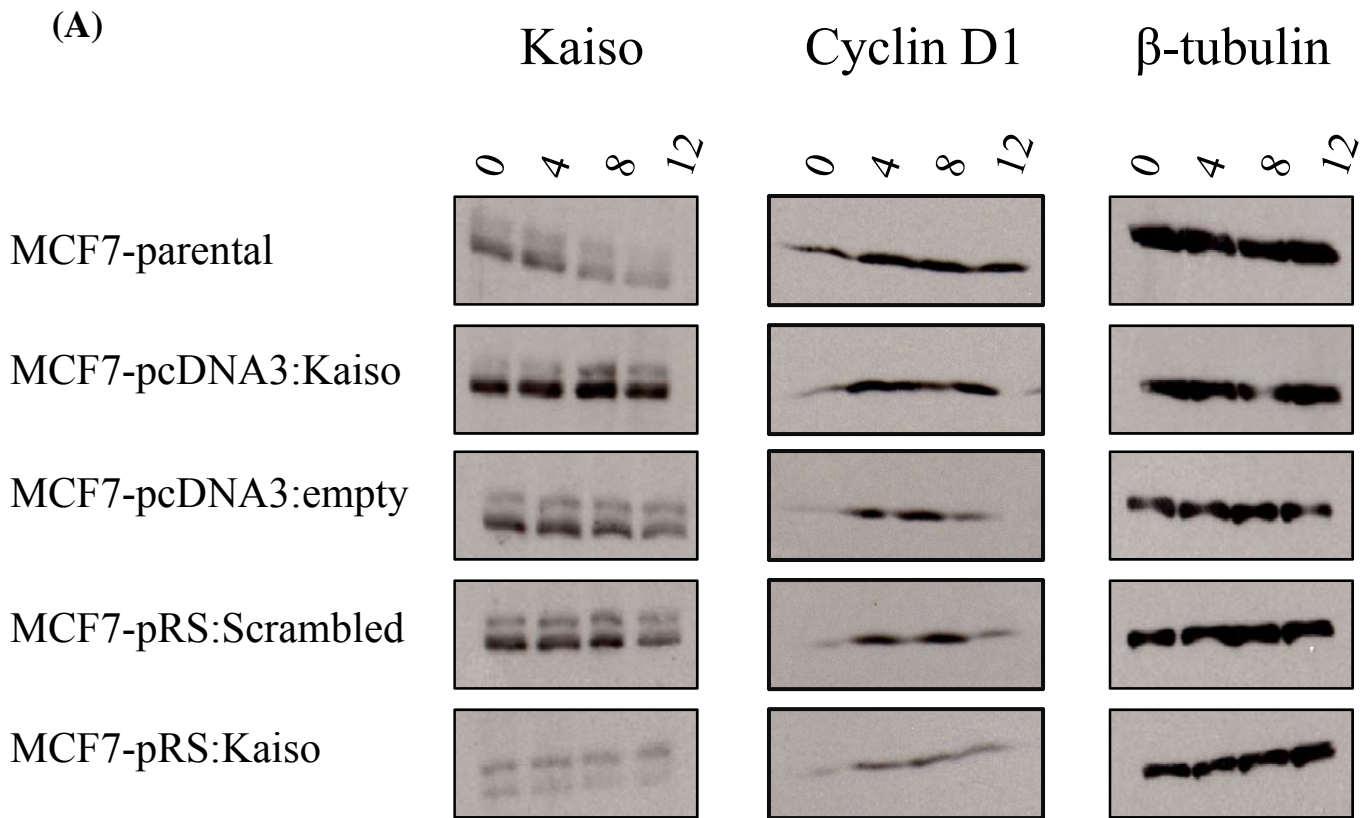
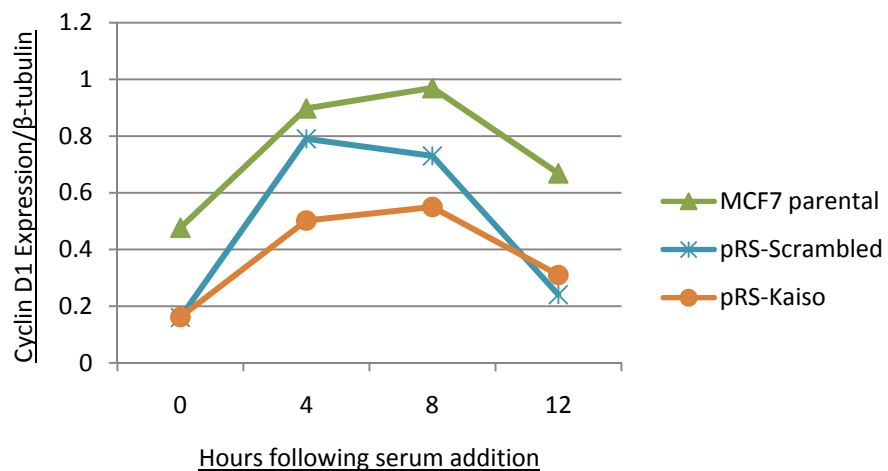


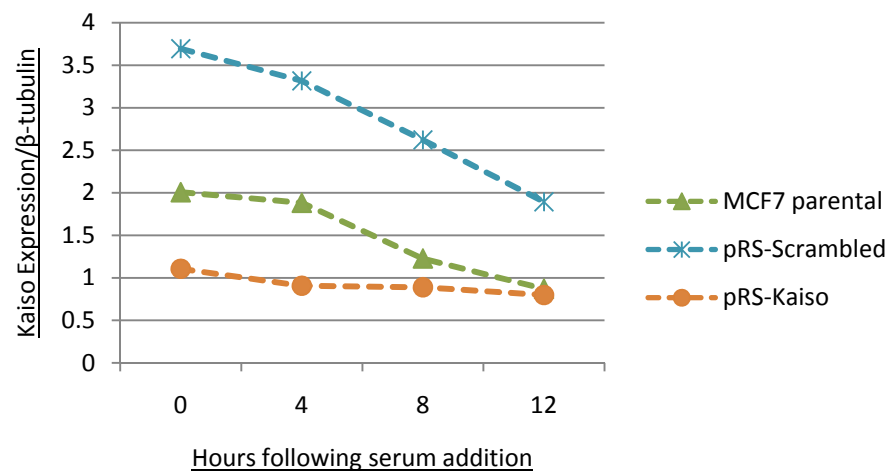
Figure 17: Cell synchronization and Kaiso misexpression analysis of Cyclin D1. MCF7 parental and misexpression cells were serum starved for 24 hours to synchronize them and serum was reintroduced. Protein was harvested at 0, 4, 8 and 12 hours post serum readdition. (A) WB analysis of protein lysates at 0, 4, 8 and 12 hours following serum reintroduction. (B) Densitometry analysis of WB bands was performed to show the changes in protein level relative to β -tubulin expression.

B)

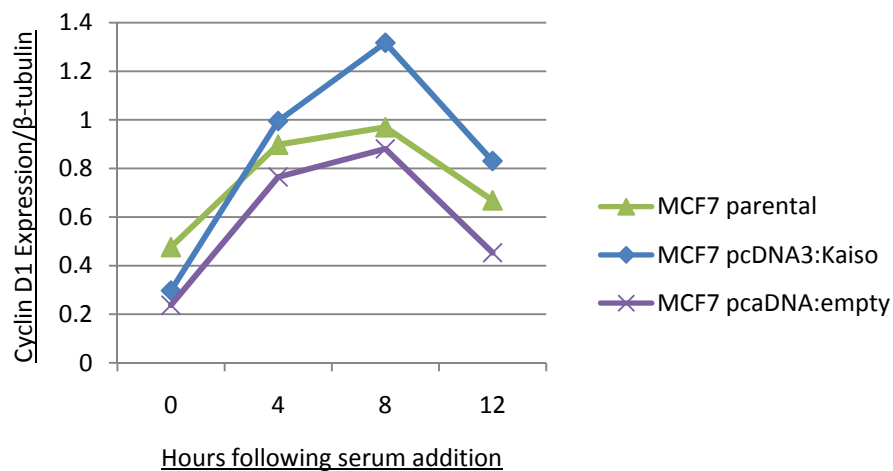
Cyclin D1 expression with Kaiso depletion



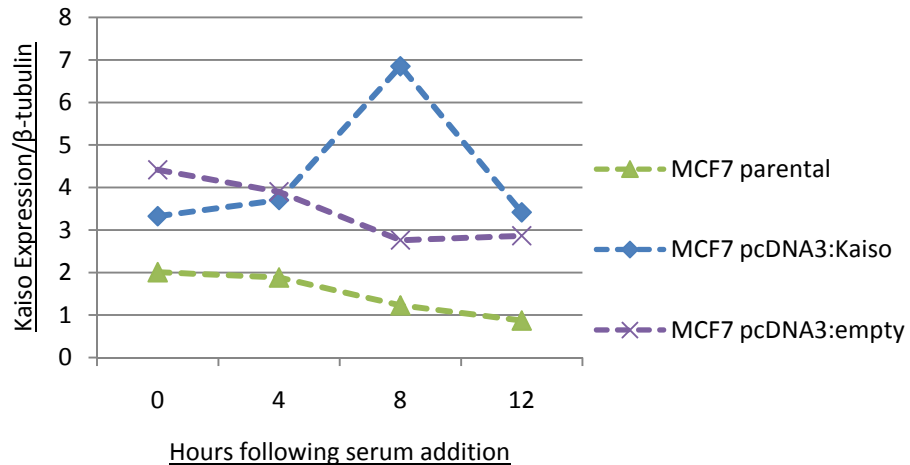
Kaiso expression with Kaiso depletion



Cyclin D1 expression with Kaiso overexpression



Kaiso expression with Kaiso overexpression



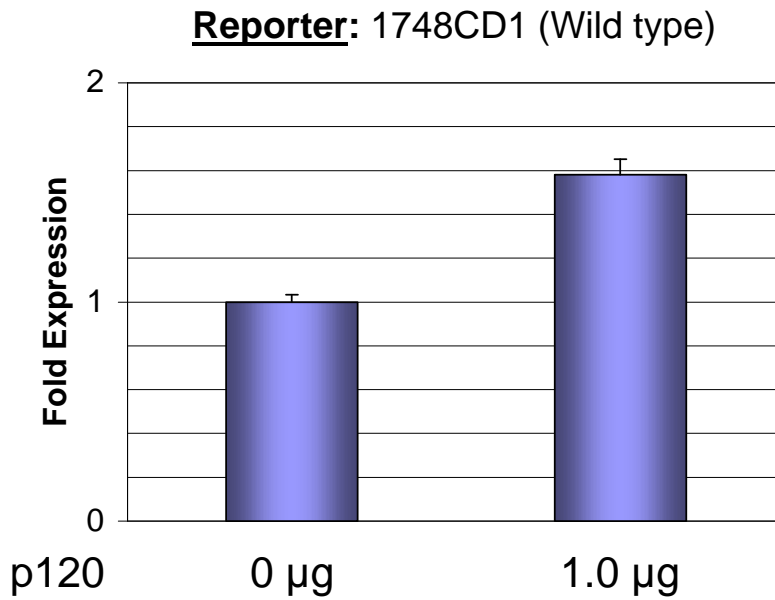


Figure 18: p120 activates the *cyclin D1* promoter. To assess whether p120 could regulate the *cyclin D1* promoter, p120 was co-transfected with the 1748CD1 luciferase reporter construct and cells were subsequently assayed for luciferase expression. Repeated experiments demonstrated that p120 activates the *cyclin D1* promoter 1.5-fold.

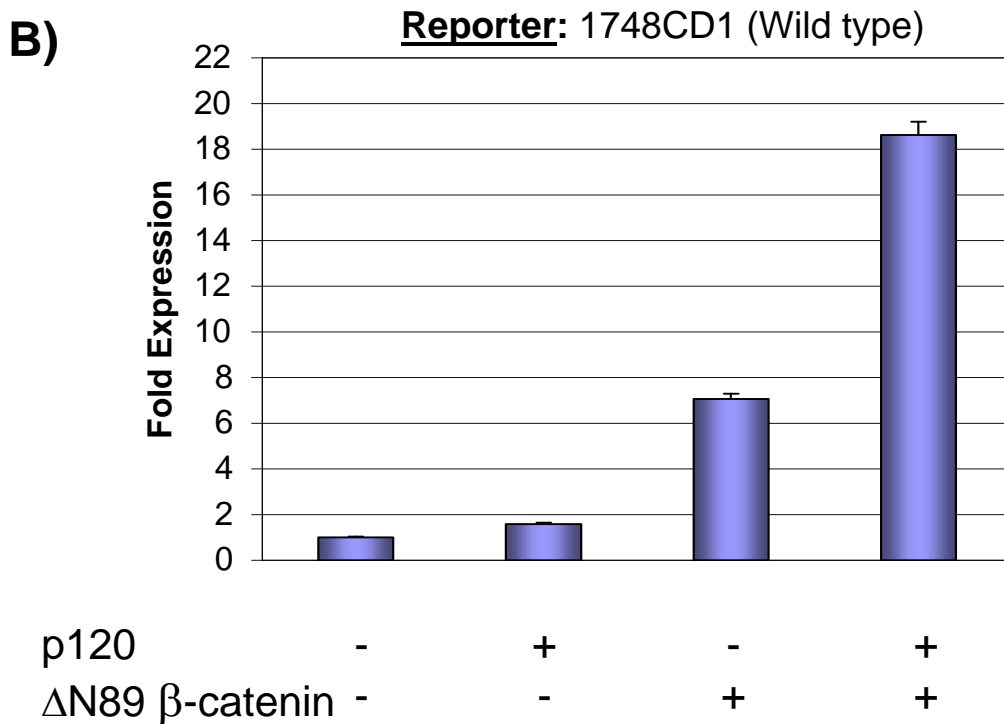
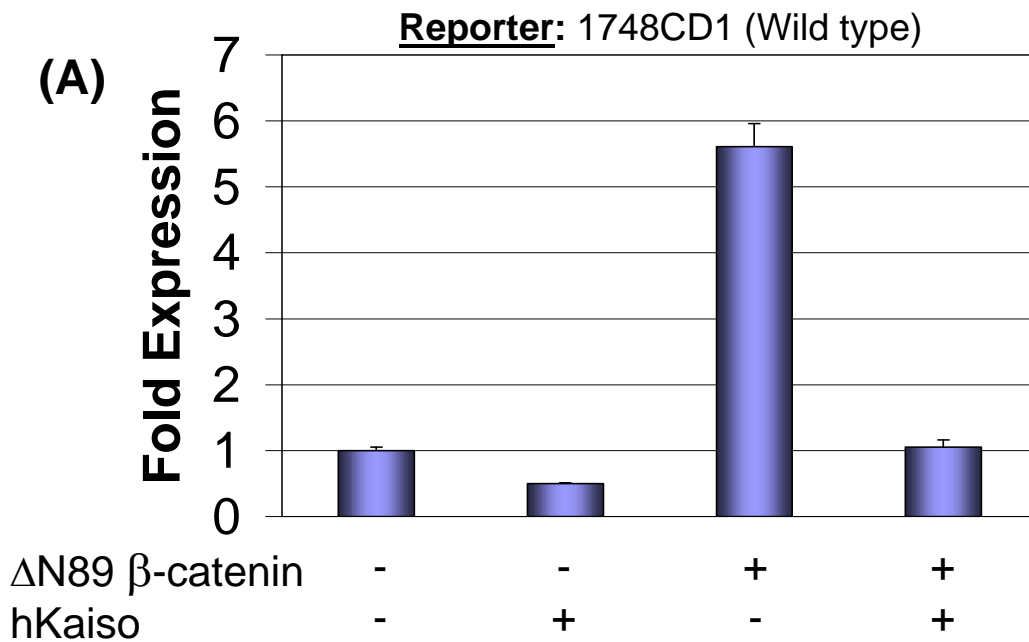


Figure 19: Kaiso and p120 modulate β -catenin mediated activation of the *cyclin D1* promoter. p120/Kaiso are reminiscent of β -catenin/TCF, well-known regulators of *cyclin D1*, leading us to believe that they may synergize or antagonize β -catenin regulation of *cyclin D1*. To investigate this the wild type 1748CD1 partial reporter construct was co-transfected with Kaiso, p120, and an N-terminally truncated and constitutively active β -catenin expression construct, Δ N89. (A) β -catenin activated the *cyclin D1* promoter 5-6-fold, however Kaiso inhibited this activation and brought luciferase expression back to basal levels. (B) Conversely, p120 enhanced β -catenin activation of the *cyclin D1* promoter to double its normal level.